J Pineal Res 1993;15:88–103
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Journal of Pineal Research
ISSN 0742-3098

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New sensitive serum melatonin radioimmunoassay employing the Kennaway G280 antibody: Syrian hamster morning adrenergic response

Vaughan GM. New sensitive serum melatonin radioimmunoassay employing the Kennaway G280 antibody: Syrian hamster morning adrenergic response. J. Pineal Res. 1993:15:88–103.

Abstract: A new procedure with the G280 antibody of Kennaway provides an assay for circulating melatonin (aMT) with a sample volume (200 μl), an analytic (0.33 pg/ml) and functional (0.62–0.80 pg/ml) detectability, a 50% displacement dose (6.4 pg/ml), a Kd (0.657 pM), and measured circulating daytime levels lower than reported for previous procedures, and 100% assay recovery. The normal daytime range in adult human and Syrian hamster serum was 0.4–4 pg/ml. The pattern of fall of the nocturnal surge of Syrian hamster serum aMT near the time of lights-on was unaltered by extended darkness. Isoproterenol (ISO) injection 1 hr after lights-on, when aMT had reached daytime levels, raised serum and pineal aMT dramatically 2 hr postinjection. The same dose of ISO injected 4 hr into light produced only a small detectable increase. Novel extension of nocturnal darkness did not affect the responses to ISO. Thus, when they are allowed to occur at the usual time on a 10-hr dark schedule, both the fall from the nocturnal aMT surge and the subsequent loss of pineal beta-adrenergic responsiveness in this species occur endogenously (probably entrained) rather than from gating by acute effects of morning light. Changes in daytime serum aMT consistent with concomitant changes in the pineal can be measured with a sufficiently sensitive radioimmunoassay.

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Key words: pineal—serum—melatonin—assay validation—Syrian hamster—isoproterenol—adrenergic response

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Received April 27, 1993;
accepted June 23, 1993.

Introduction

Lack of generalized catecholamine responsiveness characterizes the preterminal condition of nonsurviving burned and other critically ill patients with multi-organ failure. We have been interested in characterizing melatonin (acetylmethoxytryptamine) secretion in an animal model (Syrian hamster) that exhibits a normal daytime fall of catecholamine responsiveness in the pineal gland similar to that in normal humans [Vaughan, 1986, 1989]. In both species, beta-adrenergic activity stimulates melatonin (aMT) secretion at night.

Acute exposure of Syrian hamsters in vivo or of their pineals in vitro, either to the transmitter norepinephrine (NE) in the presence of a blocker of protective reuptake or to the beta-agonist isoproterenol (ISO), which does not require prevention of uptake, raised pineal aMT content (in vivo) or aMT secretion from pineals into the medium (in vitro) when the agonist was applied one time during the second half of the 10-hr dark phase, but not during the light phase [Vaughan et al., 1986a; Reiter et al., 1987; Vaughan and Reiter, 1987]. We reported [Reiter et al. 1987] that a single intraperitoneal injection of 1 μg/g NE (after reuptake blockade) in this species failed to stimulate pineal aMT content when given 2 hr into the light phase. However, pineals taken ¾ hr into the light phase responded to NE in the incubation medium. NE-induced aMT secretion into the medium was markedly diminished in pineals taken 2¾ hr into light [Vaughan et al., 1986a].

In order to assess the Syrian hamster pineal

beta-adrenergic response in the early morning, 1 μg/g ISO, which is effective at night [Vaughan and Reiter, 1987] and does not require the complication of reuptake blockade, was injected subcutaneously. Because we wanted to observe daytime circulating aMT, which is too low to be assessed with current aMT assays, we developed a new RIA procedure using the previously reported G280 antibody of Kennaway to achieve sufficient sensitivity.

Materials and methods

Assay of serum aMT

G280 antibody was supplied by D. Kennaway, Department Obstetrics and Gynaecology, University of Adelaide, Adelaide, South Australia. Its specificity profile and use for radioimmunoassay (RIA) of circulating aMT have been reported [Kennaway et al., 1982; Earl et al., 1985]. That procedure involved a sample volume of 0.5 ml extracted with dichloromethane/hexane in the presence of borate buffer, an assay buffer containing bovine albumin and globulin at pH 7.4, ^{3}H-aMT tracer (specific activity 87 Ci/mmol), final G280 antibody dilution of 1:480,000, final reaction volume of 0.8 ml, incubation for 15 min at 37°C, immersion in an ice bath for 1 hr or more, and charcoal separation of the bound and free tracer. Instead (see Table 1), we use a sample volume of 0.2 ml extracted with chloroform, alkaline and neutral H_2O washes, a pH 7.0 buffer with gelatin, ^{125}I-aMT tracer (2-iodo-aMT, specific activity 2200 Ci/mmol), final G280 antibody dilution of 1:2,880,000, final reaction volume of 0.6 ml, incubation for 20 hr at 4°C, separation of the bound tracer using a precipitating antibody (and sheep gamma globulin carrier) present during the G280 antibody incubation, and final precipitation with ammonium sulfate.

Though the G280 is caprine, omission of the equine anti-sheep second antibody severely reduces tracer precipitation. With unextracted buffer reaction medium, second antibody and carrier quantities were optimized for tracer precipitation (up to 94% of tracer counts with larger nonroutine amounts of G280 present). Binding characteristics of the G280 antibody in unextracted buffer were explored by varying the concentrations of G280 and tracer. Bound (precipitated) counts were corrected for nonspecific binding, which was less than 2% of the free.

With use of the reagent concentrations in Table 1, procedural recovery of aMT after extraction was assessed by comparison of results with those from standard curves not extracted with chloroform and not exposed to petroleum ether (beginning at step 10 after volume adjustment), or extracted with the eluate frozen on dry ice for decanting the petroleum ether and then thawed and retained in the same tubes for continuation of the procedures. The latter sequence (extraction without transfer to different assay tubes) was termed "extracted" and the previous sequence was termed "unextracted." The routine procedure (Table 1) used for this recovery comparison and at other times for assessment of assay performance and determination of unknowns was termed "extracted and transferred," and was applied to standards and all samples when present.

TABLE 1. G280 Assay procedure

1. Extract 200 μl melatonin standards (triplicates from < 1 to 100 pg/ml in buffer[a]) and samples (duplicates) with 2 ml $CHCl_3$ in 12 × 75 mm glass tubes (VCA).[b]
2. Wash $CHCl_3$ with 200 μl 0.1 N NaOH (VCA).
3. Wash $CHCl_3$ with 200 μl H_2O (VCA).
4. Add 200 μl H_2O (only centrifuge and aspirate).
5. Evaporate $CHCl_3$ at room temperature in a vacuum centrifuge (1–2 hr), replenish with N_2.
6. Elute overnight at 4°C with 500 μl buffer after vortexing.
7. Wash eluate with ml petroleum ether: vortex, let settle at 4°C 1 hr, aspirate organic phase.
8. Vortex eluate, let organic traces evaporate 1 hr at 4°C.
9. Transfer 400 μl eluate to assay tubes (we use glass tubes) in ice bath.
10. Add 50 μl sheep gamma globulin (Sigma 15131) from a solution of 0.05 mg/ml in buffer.
11. Add 50 μl G280 diluted 1:240,000 in buffer; final incubation dilution 1:2,880,000.
12. Add 50 μl ^{125}I-melatonin (New England Nuclear, 2200 Ci/mmole, approximately 4000 cpm) in buffer.
13. Add 50 μl donkey anti-sheep-gamma globulin (Fitzgerald 40-DS40) after prior dilution 1:10 in buffer (adjust dilution for maximum tracer precipitation).
14. Vortex, incubate at 4°C for 20 hr.
15. With tubes at 4°C, add 2 ml of cold (4°C) 17.5 g/dl ammonium sulfate in H_2O (important to keep tubes at 4°C until centrifuged).
16. Incubate 30 min in 4°C bath.
17. Centrifuge 30 min at 4°C, 2,000*g*.
18. Decant supernatant.
19. Count gamma scintillations from precipitate radioactivity to 10,000 counts/tube or 10 min, express this as cpm.

[a]Buffer: 0.01 M (sodium) phosphate, 0.9% NaCl, 0.1% gelatin, 100 mg/L thimerosal, pH 7.0.
[b]VCA: vortex, centrifuge, aspirate aqueous layer.

Serum samples used in assessment of assay performance were pools from Sprague-Dawley rats, Syrian hamsters, or humans, or were from individual healthy adult humans or from one man following successful irradiation of a germinoma that had previously been present at two sites (pineal and anterior hypothalamus).

The analytic least detectable value (ALD) in buffer was the aMT concentration in a given assay obtained at 2 SD below the mean cpm of 6 or 9 zero standard replicates. The functional least detectable value (FLD) was the aMT concentration below which the between-assay coefficient of variation

was above 20%, determined from different human, hamster, and rat serum samples, each measured in one duplicate pair of tubes over several assays. The FLD provides a more conservative index of the lower end of the useful range of an assay in its practical performance with serum samples [Spencer et al., 1990; Nicoloff and Spencer, 1990].

Experimental design

Young adult male Syrian (*Mesocricetus auratus*) hamsters were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained (three to five per clear plastic cage) in the experimental light:dark cycle of 14/10 hr (lights-off 2000, on 0600) for at least 2 weeks prior to the study with access to tap water and laboratory chow ad libitum. Injections were given subcutaneously in a volume of 0.15 ml. Groups of eight to ten hamsters were killed by guillotine at 2000 (just before lights out), 2200, 2400, 0400, 0550, 0616, 0620, 0700, 0900, 1000, and 1200. Sampling in the dark was done with the aid of a 25-watt lamp behind a Kodak 1A safe light red filter. The animals sampled at 0900 had been injected with physiologic saline (0.9% NaCl) at 0700, and those sampled at 1200 had received NaCl at 1000. Other groups were sampled at 0400 (after 1 μg/g propranolol injected at 0200); at 0900 (after 1 μg/g ISO injected at 0700); and at 1100, 1200, and 1300 (after ISO injected at 1000). On another day, the portion of the above protocol from 0550 onward was repeated with the exceptions that at 0600, on only the morning of this part of the experiment, the lights were not turned on (i.e., novel morning darkness extended from the night through all sampling), and that the first sampling after 0600 was at 0620. Trunk serum was saved at −60°C. Pineal glands from animals sampled at 0900 and beyond were saved at −60°C for assay using the Rollag antibody as described previously [Vaughan et al., 1985], with an ALD of 5 pg/pineal.

Analyses

Assay results were calculated with a four-parameter logistic regression of precipitated standard cpm (including zero aMT) with pre-extraction aMT expressed in pg/ml. The relevant formulas for regression (predicted cpm) and calculations are shown in the footnotes to Table 2. Calculations (P1D), regressions (PAR, P6D), analyses of variance (P7D) and covariance (P1V), and t-tests with the Bonferroni correction for multiple nonindependent comparisons where applicable (P7D) were performed with the indicated programs of the BMDP statistical package [Dixon, 1990] on a VAX 4000 computer system.

Results

G280 assay performance

The high affinity of the G280 antibody obtained under these conditions suggests that use of constant concentrations of tracer and antibody in a low range (Fig. 1, Table 3) should provide an assay with high sensitivity. Rearrangement of the elements of the mass law equation (total tracer and total nontracer ligand expressed separately, and equation elements combined into the tracer binding proportions [B_0, B/B_0, see Table 2]) allows prediction of the aMT concentration that might be required for a given B/B_0, with the routine concentrations of tracer and antibody (the latter indexed by the B_0), as a function of the Kd applying to both tracer and nontracer ligand (molar in the reaction mixture):

$$[\text{aMT}] = \left(\frac{1}{B/B_0} - 1\right)\left[^{125}\text{I-aMT}\right] + \frac{Kd}{(1 - B_0)(B/B_0)} - \frac{Kd}{1 - B_0(B/B_0)}.$$

With use of AMT-free buffer, B_0 averages about 51% for the amount of tracer (e.g., about 4,129 cpm) and of antibody to be used in the assay. The SD of the B_0 replicates (as B/B_0, i.e., each divided by the mean) is usually about 2.5%, giving a mean (100%) minus 2 SD as 95% B/B_0. The above formula with the Kd thus gives a theoretical ALD prediction of about 0.24 pM in the reaction mixture or about 0.21 pg/ml in an unextracted sample, without use of standard curve observations. The ALD, found to be in this general range (Table 2) from actual standard curves, lends credence to the antibody affinity determination and the high sensitivity of the assay.

Incorporation of (nonradioactive) aMT did not not alter the binding slope (Fig. 1) or the binding profile with the higher ligand concentrations (Fig. 2) needed for an asay, suggesting that G280 binds ^{125}I-aMT and aMT with virtually identical characteristics throughout the concentration range of their use in the assay. Figure 3 shows the typical distribution of tracer quantity expressed as observed total cpm/tube among assays (constant in a given assay) and the resulting B_0. The negative relationship of B_0 with varying total cpm, the latter mainly a function of variable time for radioactive decay following preparation in buffer, is consistent with the supplier's assertion that ^{125}I-aMT undergoes catastrophic decay, leaving nonradioactive products without apparent immunoactivity in this system. The proportion (about 94%) of cpm precipitated in the presence of nonroutine excess amounts of G280 does not

TABLE 2. Assay parameters, calculations and characteristics (20 assays; standard and sample volume 0.2 ml, standards ranging from < 1 to 100 pg/ml)

	Mean	SE	Comment
Total cpm[a]	4129	205	total per tube
Logistic parameters			
a	2151	77	fitted max cpm (zero aMT)[b]
b	70	6	fitted min cpm (at infinite aMT)
c	6.44	0.11	fitted ED^c_{50} (pg/ml)
d	1.0332	0.0109	slope factor
Nonspecific binding			
NSB_0 (%)	1.90	0.11	cpm/total (without antibody)
NSB_b (%)	1.74	0.14	b/total (for infinite aMT)
ALD (pg/ml)	0.33	0.03	for zero-aMT cpm mean − 2 SD
FLD (pg/ml)	[0.62 − 0.80]		aMT limit for BACV[d] = 20%
B/B_0[e] (%)	95.6	0.4	for aMT = 0.33 pg/ml (ALD)
B/B_0 (%)	91.8	0.3	for aMT = 0.62 pg/ml (FLD)
B/B_0 (%)	89.6	0.3	for aMT = 0.80 pg/ml (FLD)
ED_{05} (pg/ml)	0.38	0.01	for B/B_0 = 95%
ED_{10} (pg/ml)	0.77	0.02	for B/B_0 = 90%
ED_{20} (pg/ml)	1.69	0.04	for B/B_0 = 80%
ED_{50} (pg/ml)	6.44	0.11	for B/B_0 = 50%
ED_{80} (pg/ml)	24.75	0.57	for B/B_0 = 20%
ED_{90} (pg/ml)	54.53	1.71	for B/B_0 = 10%
ED_{95} (pg/ml)	113.11	4.58	for B/B_0 = 5%

[a]cpm = counts per minute in precipitate, unless specified as total. Predicted cpm = $\frac{a-b}{1+\left(\frac{aMT}{c}\right)^d} + b$.

[b]aMT = melatonin in pg/ml before extraction. Predicted aMT = $c\left(\frac{1}{B/B_0} - 1\right)^{1/d}$.

[c]ED, effective dose.

[d]BACV, between-assay coefficient of variation.

[e]$B = \frac{cpm - b}{\text{total cpm}}$. $B_0 = \frac{a-b}{\text{total cpm}}$. Predicted $B/B_0 = \frac{1}{1+\left(\frac{aMT}{c}\right)^d}$.

appear to change during the time of use, indicating stable attachment of the ^{125}I atom to the aMT prior to decay.

Table 2 gives routine assay logistic parameters and characteristic relationships between aMT concentration and tracer binding inhibition. Standards from <1 to 100 pg/ml produce a range of displacement from about 5 to 95%, with 50% displacement at 6.44 pg/ml (the ED_{50}). The analytic (ALD) and functional (FLD) least detectable concentrations produce displacements of about 5% and 10% respectively. An assay tracer binding profile (Fig. 4) shows essentially parallel binding of dilutions of serum.

Figure 5 shows that omission of chloroform extraction of a human day serum sample results in a false high value of 17 pg/ml and nonparallel dilution, compared to 2.2 pg/ml with parallel dilution after extraction. (Nonspecific crossreactants are reflected by nonparallelism only if the former happen to have different antibody binding charcteristics from those of aMT over changing concentrations in the relevant ranges.) Of importance, there is no detectable loss of aMT itself in the extraction, washing, and transfer procedures (Fig. 6). Thus, standards and samples are routinely extracted to minimize nonspecific serum crossreactivity, provide a common procedural base for any factors potentially affecting variation in results, and allow assay recovery from serum that is not significantly different from 100% after dilution or after addition of known amounts of aMT (Fig. 7). For human serum samples ranging 1–80 pg/ml aMT, there were whole blood aliquots collected simultaneously in heparin (seven samples, 20–80 pg/ml aMT) or potassium EDTA (12 samples, 1–46 pg/ml aMT) for comparison of plasma values (data not shown). Analysis of covariance showed no difference in either series of plasma values from those of serum.

Figure 8 shows the assay coefficients of variation. The serum between-assay coefficient of variation is <20% for aMT above 0.62–0.80 pg/ml and

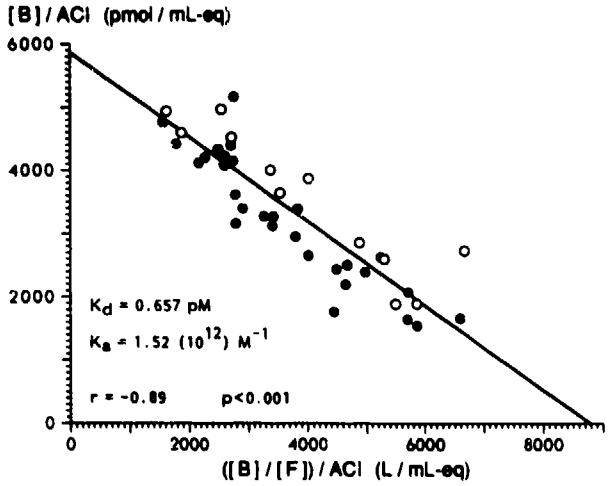

Fig. 1. Scatchard analysis of G280 antibody. Data are means of duplicates or triplicates. [B] (concentration of bound ligand or bound antibody binding sites) and [F] (concentration of free ligand) are in pM of reaction mixture. ACI (antibody concentration index) is in ml equivalents of undiluted G280 goat serum (ml-eq) per liter of reaction mixture. The Kd (dissociation constant = −slope) = 1/Ka (association constant). The Bmax (y intercept) is 5800 pmol/ml-eq. Closed circles, tracer ^{125}I-aMT as the only ligand present. Open circles, nonradioactive aMT as 18–91% of total ligand (90–91% for 8/12 samples). For the open circles, [B] was taken as bound tracer augmented by the amount of aMT in the fraction (of total nonradioactive aMT present) equivalent to the fraction of tracer that was bound, allowing a different affinity for aMT to be manifested as an altered slope. Analysis of covariance detected no difference between slopes with and without aMT. The slope from the pooled data was used for the plot and the Kd. Ranges in the data: total ligand (0.80–5.4 pM), total antibody binding sites (0.40–6.03 pM), total ligand/antibody sites ratio (0.33–5.70), ligand bound (14–86%), antibody sites bound (28–82%).

less than 10% for aMT above 2 pg/ml. The FLD is in the range of 0.62–0.80 pg/ml, in comparison with the ALD of 0.33 pg/ml.

The assay can detect the human nocturnal aMT surge (Fig. 9) and appears to detect normal daytime values, which range 0.4–4.0 pg/ml (Fig. 10). The latter were all above the ALD and most were above the FLD. In contrast, all (including nocturnal) values from the patient with postirradiation pineal and hypothalamic atrophy (and hence likely reduced or absent pineal function) were below the ALD (Fig. 10).

Syrian hamster experiment

Subcutaneous ISO injection (1 μg/g) after 1 hr of morning light (when serum aMT has already reached the daytime level) is capable of elevating mean circulating aMT by at least five-fold above the daytime mean of just less than 2 pg/ml. Delaying the injection until 4 hr into the light phase produced a markedly smaller, though still significant, response (Fig. 11). Assay of the injectate did not detect aMT crossreactivity. Neither the baseline aMT values nor the postinjection responses to ISO were altered by extension of nocturnal darkness into the morning experimental time. The lower limit (0.7 pg/ml) of the mean ± 2 SD control range (daytime values after 0800) is essentially the same as the FLD (Fig. 11). Figure 12 shows that in these animals on this 10-hr dark schedule, a large part of the fall of the nocturnal serum aMT surge occurred near the expected time of lights-on, even if the lights did not come on. An injection of propranolol prevented or inhibited most of the rise otherwise seen at 0400.

Pineal aMT content, available in the present experiment from the animals sampled after 0800, showed a large rise from ISO injected after 1 hr of morning light and a smaller (but still significant) rise from injection delayed until 4 hr into the light phase (Fig. 13). This pattern and the lack of detectable influence from novel extension of darkness resemble the findings in serum in the same animals. The responses of pineal aMT 2 h after 1 μg/g ISO injected either after brief exposure to light at night [Vaughan and Reiter, 1987] or at 1 h into the expected time of morning light are similar (Fig. 14), but contrast with the small response after 4 h of light and the absent response by the end of the light phase [Vaughan and Reiter, 1987].

Discussion

The magnitude of circulating daytime aMT levels has so far been uncertain, because values have usually been below the assay detection limits or have varied widely among assays even when above the quoted least detectable. RIA sensitivity can be indexed by several parameters including the ALD, the ED_{50} (effective dose at 50% tracer displacement), and the Kd or Ka of the antibody and melatonin under the conditions of the assay. In reports that define the cited estimates of "sensitivity" or "detectability," this has usually been the curve-fitted amount of aMT at tracer displacement of 2 SD below the mean of zero-standard replicates, or, occasionally, the amount at 5% displacement of tracer, which may provide a similar estimate. In any case, even if the precise determinant was not given, the estimates reported are herein termed the ALD. They represent what was perceived, within a given assay run and its analytic constraints, as the minimum aMT required to give a consistent analytic response in buffer or special (sometimes stripped) serum matrix. Such media for standards are constant and/or lack many components that may vary among samples of native serum or plasma. The ALD and

TABLE 3. Tracer and aMT concentrations before extraction and in assay reaction mixture (with 2.0 pM antibody binding sites)

Identification	Concentration in standard or sample before extraction		Amount per assay tube		Concentration in reaction mixture
	pg/ml	pM	pg	pmol	pM
Total cpm (e.g., 4129)			0.265[a]	0.00115[a]	1.91[a]
aMT at ALD	0.33	1.42	0.0528	0.000228	0.379
aMT at FLD	0.62	2.67	0.0992	0.000428	0.713
aMT at FLD	0.80	3.45	0.128	0.000552	0.920
aMT of typical lowest standard	0.50	2.16	0.0800	0.000345	0.575
aMT at ED_{50}	6.44	27.8	1.03	0.00444	7.40
aMT of highest standard	100	431	16.0	0.0690	115

[a]With observed total activity (4,129 cpm) subsequently corrected to 94% as ^{125}I-aMT, the logistically projected fraction precipitated at infinite G280 concentration, applied in determination of the binding constants in Figure 1, but not in the assessment and use of the assay; the gamma counter efficiency for the glass tubes used was 70%; pg tracer given as aMT equivalent; tracer quantity not included for aMT (the other entries) under amount per tube or in reaction mixture; this amount of tracer, if present as aMT equivalent, would be 1.66 pg/ml or 7.16 pM in an unextracted sample.

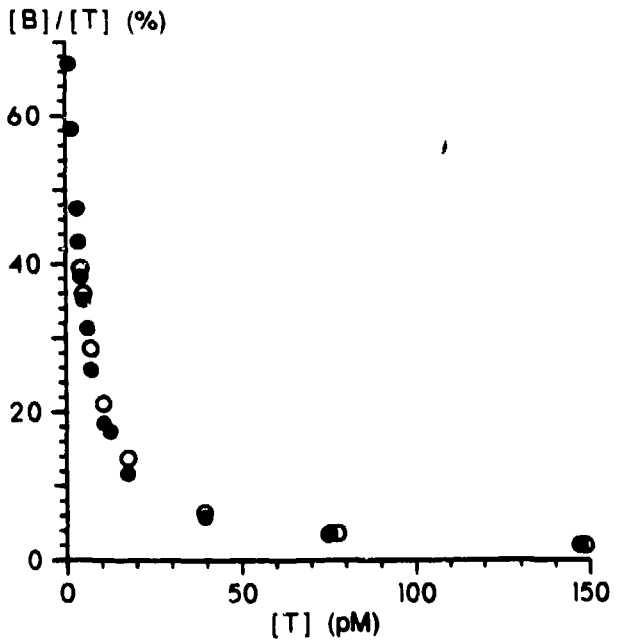

Fig. 2. Proportion of ^{125}I-aMT tracer bound as a function of total ligand concentration in the reaction mixture over a range wider than that in a typical assay standard curve with the constant G280 antibody concentration used in the assay. Open circles, constant tracer concentration typical of an assay, with added nonradioactive aMT including the range used in the assay. Closed circles, variable tracer concentration as the only ligand.

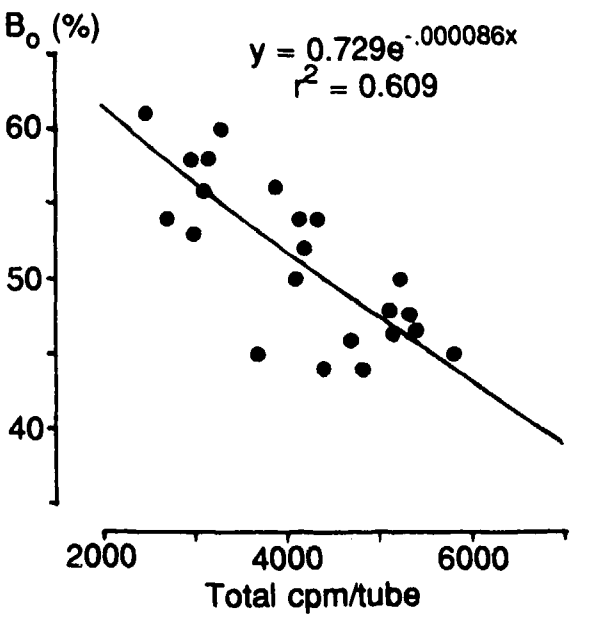

Fig. 3. Proportion of tracer cpm bound by the small constant assay quantity of G280 antibody in the absence of aMT, as a function of total tracer cpm. The data include the mean zero-standard binding in the assays summarized in Table 2. Variation in the total cpm was due largely to physical decay over variable time intervals after preparation of the tracer for use.

ED_{50} are herein expressed per ml of sample rather than per assay tube, to assess procedures as they are actually used. Expressed this way, parameters of sensitivity and detectability are worsened with smaller sample volume under conditions otherwise the same. Higher sample volumes to enhance these parameters may also increase nonspecific serum or plasma factors in the assay and interact with sample collection limitations.

Since the projected aMT value in a range near the limit of detection varies greatly for any small error in assay signal (e.g., B/B_0 where the slope is less steep), aMT values in this range, even if above the ALD, are liable to larger relative errors than values above this range. The sources of such error, usually conceived to be either systematic (nonspecific serum factors) or random, operate variably within and between given assay runs. If the between-assay

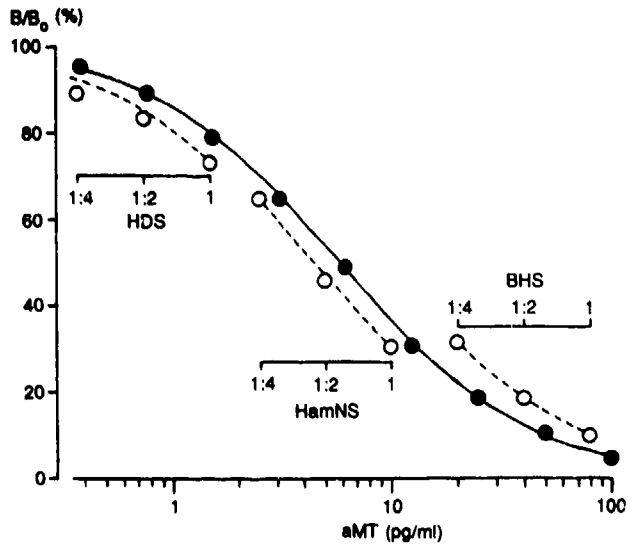

Fig. 4. Binding profile of standards in an assay run. Closed circles, observed means of standard triplicates. Open circles, dilutions of serum (means of duplicates) displaced uniformly to the right or left of the standard curve. Dashed lines, projected from undiluted serum are parallel to the fitted standard curve. HDS, pooled human day serum; HamNS, pooled Syrian hamster night serum; BHS, pooled burn human serum collected from burn patients at about 0500. Abscissa, aMT prior to extraction.

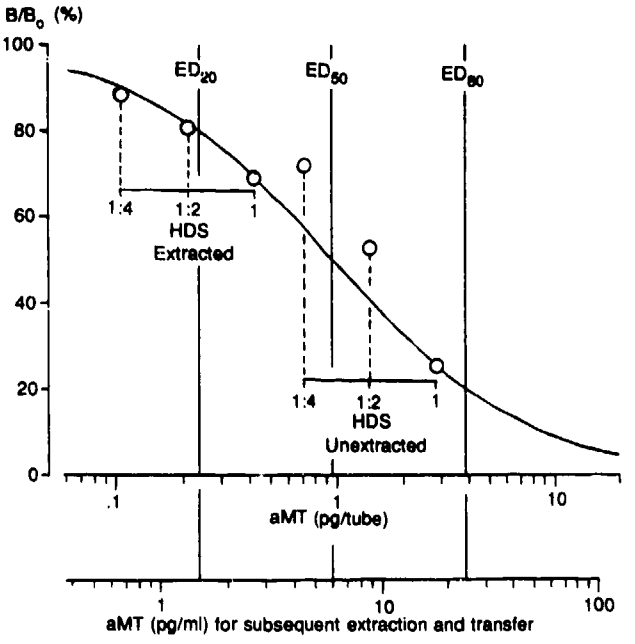

Fig. 5. Effect of omitting sample extraction. In this (extracted) assay, one set of pooled human day serum (HDS) was left unextracted: 200 µl serum placed in tubes with 300 µl buffer beginning at step 6 of Table 1, omitting steps 7 and 8. The other set of HDS aliquots was prepared in the regular fashion (Table 1). Standards are represented only by the logistically fitted line, and undiluted serum sample coordinates are placed on the fitted standard curves with serum dilutions as shown. In this case, two abscissas show aMT both as concentration and amount per tube.

(between-run) coefficient of variation (BACV) for a control serum or plasma sample in several assays is determined by using within-assay means of the same number of replicates (e.g., a single pair of duplicates) as used for determination of unknown samples, then across-assay variation for this control sample results from a composite of within- and between-assay error forces that probably also affect the unknown serum or plasma determinations. The error in any determination includes a contribution from being in a given assay run as opposed to another run even if only one run is made. This part of the error is not disclosed by within-assay parameters. The effects of nonspecific factors peculiar to native samples together with those of the random error forces become magnified (relative to the mean analyte value) in the low analyte range, and the BACV thus includes some of these influences. This allows one to determine a lower limit of analyte in serum or plasma (the FLD) below which the BACV rises above a given level (e.g., 20%) [Spencer et al., 1990; Nicoloff and Spencer, 1990], indicating the sizeable risk of measurement error, but at a more conservative threshold than indicated by the ALD with only buffer or standard matrix influences operating within assay runs. Values above the FLD (determined in serum) may more likely be taken to represent the output of the method.

Values above the FLD are not necessarily free of nonspecific error influences but the role of these influences exerted more powerfully (relative to the analyte content) at the lower range of measurement may be minimized above the FLD. Less assay specificity allows such serum influences to be exerted into higher ranges of analyte, and this might be indexed mainly or only by higher apparent analyte levels, particularly when they should be low. Thus, particularly for samples with low expected analyte content, if assay recovery for a given procedure is at least comparable to that of others, lower values likely indicate greater assay specificity. Such comparisons for daytime aMT values, which should be low, may be informative. For some of the RIA kits mentioned below, extraction of samples, but not standards, may be employed, and a difference in intra-assay precision and/or recovery between standards and samples may thus risk an overstatement of sensitivity.

The present antibody (G280) was previously used in a procedure [Earl et al., 1985] with an ED_{50} of 93 pg/ml and an ALD of 15 pg/ml on 500-µl samples, compared with 6.4 and 0.33 pg/ml on 200-µl samples in the present procedure. Large

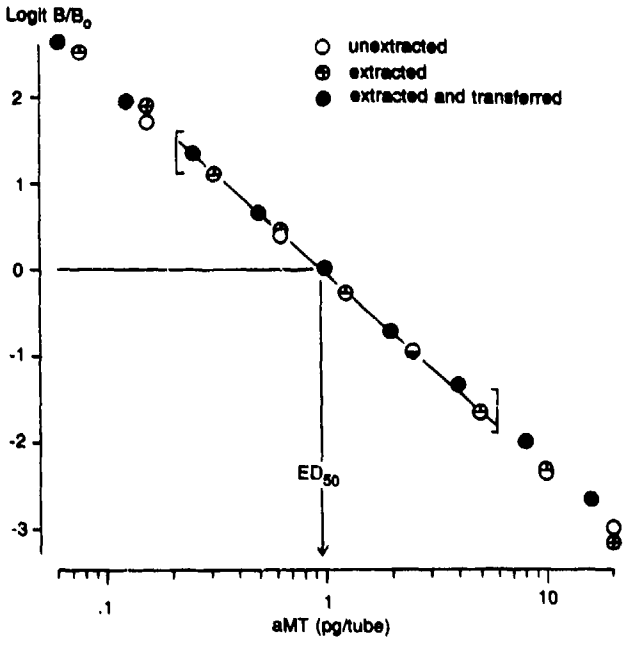

Fig. 6. Procedural recovery of standards. Symbols are means of six to nine replicates. Values surrounded by brackets were subjected to analysis of covariance showing no difference in slope or position among the three procedural groups.

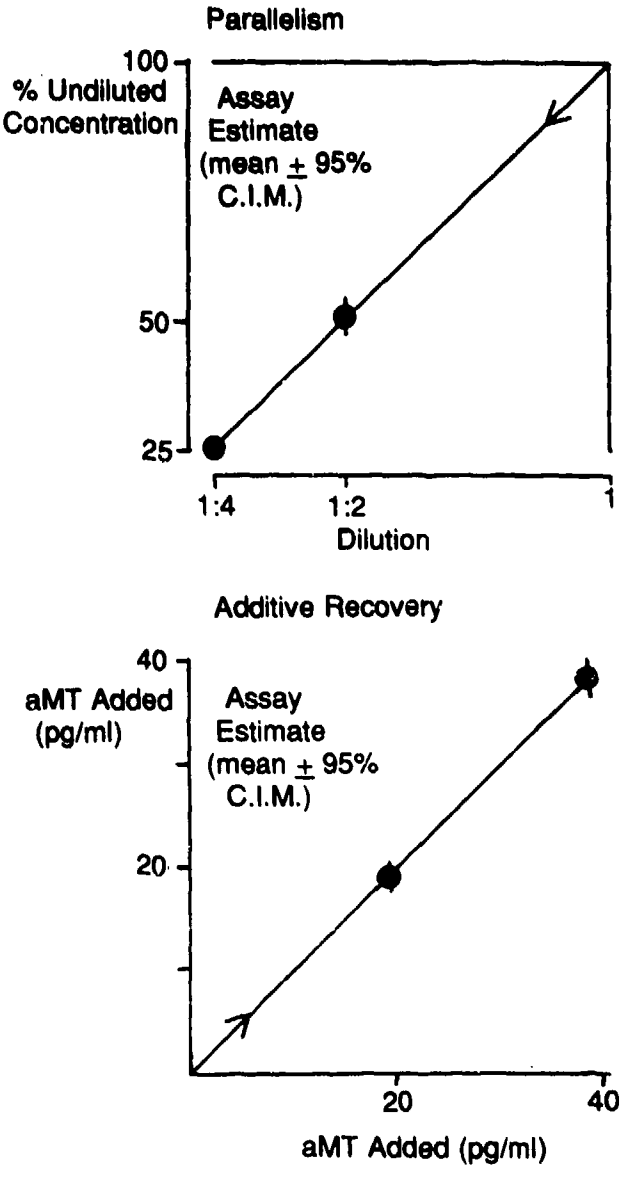

Fig. 7. Dilutional and additive serum assay recovery measured over eight assays. C.I.M., confidence interval of the mean. Diagonals: lines of identity. Starting samples: hamster night serum, 14 pg/ml, five assays and human burn serum, 54 pg/ml, three assays (top panel); hamster night serum (five assays) and human day serum, 0.9 pg/ml, three assays (bottom panel).

reductions in the amount of tracer (greater specific activity of ^{125}I vs ^{3}H label) and of antibody were possible because of the high antibody affinity and, likely, are mostly responsible for the improvement. Another procedure used previously in our laboratory employed the Rollag antibody and ^{125}I-aMT analogue tracer and was modified to enhance sensitivity (less antibody and tracer than in the original Rollag method) and specificity (petroleum ether wash) [Vaughan et al., 1985]. With a sample volume of 500 µl, the ED_{50} was 47 pg/ml and ALD 5 pg/ml. Daytime normal adult human serum samples registered a mean of about 14 pg/ml. In contrast, the present assay with a smaller sample volume gives a normal range for human day serum of 0.4–4 pg/ml and a FLD (0.6–0.8 pg/ml) well below the previous ALD, suggesting previous nonspecific serum crossreactivity in daytime samples.

A similar contrast can be made with most of the other commonly used aMT RIA's. Unless specified differently, methods are RIA and the tracer ^{3}H-aMT. One procedure [Pang et al., 1977; Arató et al., 1985] utilizes samples of 500 µl with an ED_{50} of 500 pg/ml, a reported ALD of 10 pg/ml, and human day serum values of 5–20 pg/ml. The widely used kit assay of WHB (Bromma, Sweden) [Wetterberg et al., 1978; Beck-Friis et al., 1984; Lissoni et al., 1986; Cavallo et al., 1987; Mozzanica et al., 1988] extracts 1 ml of serum and utilizes 20–80% of the buffer reconstitution so that there is potential variation in the pre-extraction concentration-based parameters. The ED_{50} appears to be 58–232 pg/ml, and ALD 11–45 pg/ml, though ALDs of 3, 8, and 16 pg/ml have been mentioned. Mean human day serum values have ranged 5–27 pg/ml. The Kd (from the reported Ka) was 3.3 (10^{-10}) M in comparison with our 6.57 (10^{-13}) M.

The widely used kit procedure of CIDtech (Hamilton, Ontario, Canada) [Grota et al., 1981; Brzezinski et al., 1988; Trinchard-Lugan and Waldhauser, 1989], with extraction of 1 ml serum, has

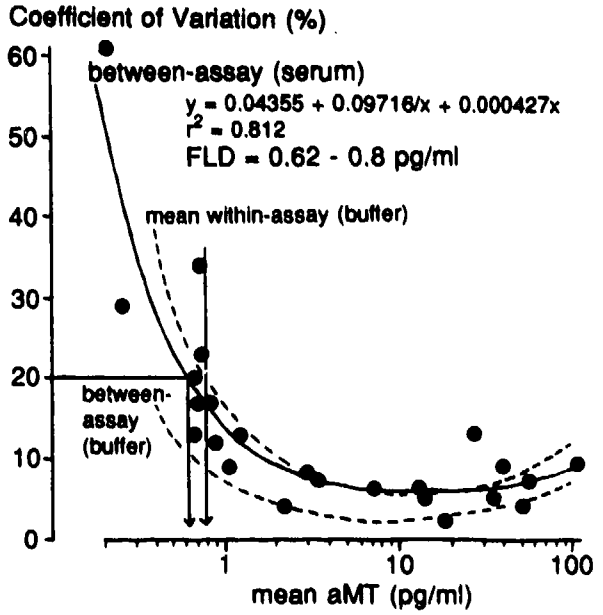

Fig. 8. Assay variation in the assays of Table 1. The functional least detectable (FLD) value is obtained from serum between-assay variation as a function of the pre-extraction concentration. Serum samples were measured in duplicate in each of three to nine assays and plotted (closed circles). The uninterrupted fitted line gives 0.62 pg/ml at 20% between-assay coefficient for serum, and 0.8 pg/ml is taken from the concentration above which all observed serum variation between assays was <20%. The buffer results are given only as their fitted curves (dashed lines) and are derived from the standards present as triplicates in each assay and whose cpm were treated as unknowns to calculate predicted aMT.

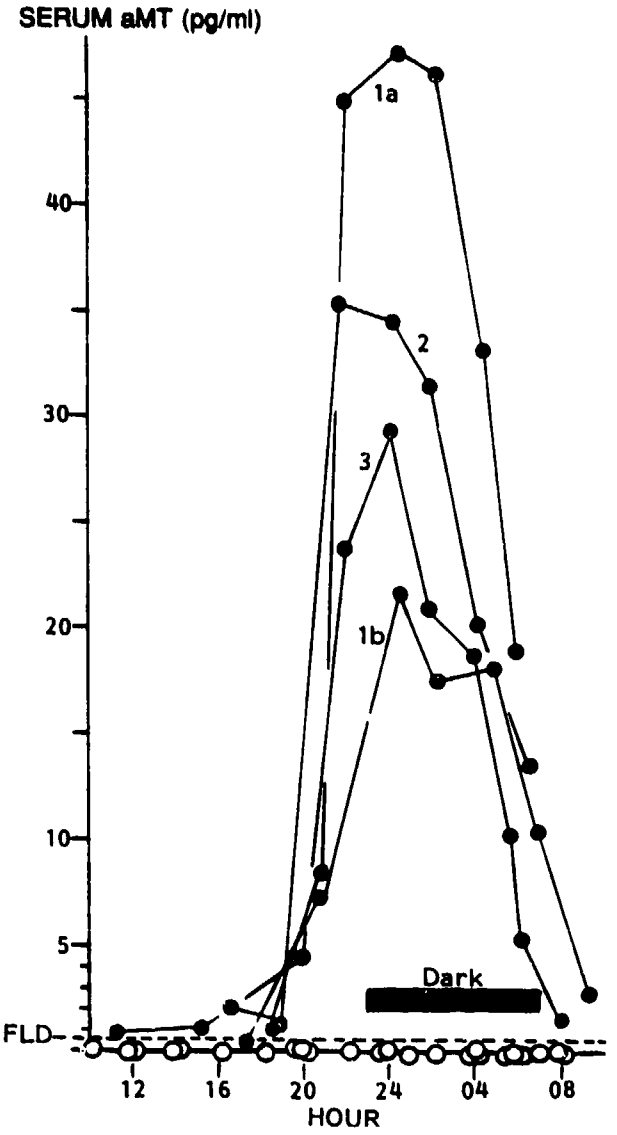

Fig. 9. Serum aMT in three healthy adult humans [ages: 40 (1a) and 47 (1b), 41 (2), 48 (3)] as closed circles. Open circles: one patient with anterior hypothalamic and pineal atrophy sampled on several occasions 2–17 years after irradiation of germinoma at age 32. FLD, functional least detectable taken as 0.62 pg/ml.

involved an ED_{50} of 50–60 pg/ml, and ALD of 5–14 pg/ml, and human day serum results centering around 5 to 26 pg/ml. In one report [Trinchard-Lugan and Waldhauser, 1989], three BACV values were given (from 16 to 26%) in a pattern suggesting a FLD of 34 pg/ml. Higher numbers of within-assay replicates of the BACV control samples might tend to lower the BACV by reducing the within-assay contribution to the BACV. If such replication is greater than for the unknown samples, then the BACV (and the FLD) might be underestimated for the purpose of assessing potential error in unknown samples. Though the within-assay replication for BACV samples was not given, the large number of assays (apparently 32) suggests some fidelity in the above tentative FLD estimate as a lower limit. Conservatively, it would appear higher than the present 0.62–0.80 pg/ml from within-assay duplicates.

The widely used Stockgrand Ltd. kit assay (Guildford, Surrey, UK) [Fraser et al., 1983a; Fraser et al., 1983b; Bojkowski et al., 1987; Strassman et al., 1987; Thompson et al., 1988; Strassman et al., 1989; Rivest et al., 1989; Shanahan and Czeisler, 1991] has the advantage that it does not require sample extraction. The sample volume is 500 µl and the ED_{50} originally [Fraser et al., 1983a] was about 120 pg/ml, though it may be less with the use of the lower amount of antibody recommended subsequently. The ALD has been in the range of 5–10 pg/ml. Human day serum is often stated to be below the ALD, though means of up to about 15 pg/ml have been reported. In one report [Bojkowski et al., 1987], BACV (10–21%) was given for three aMT levels in a pattern compatible with a FLD of about 25 pg/ml. Without knowledge of the extent of within- and between-assay replica-

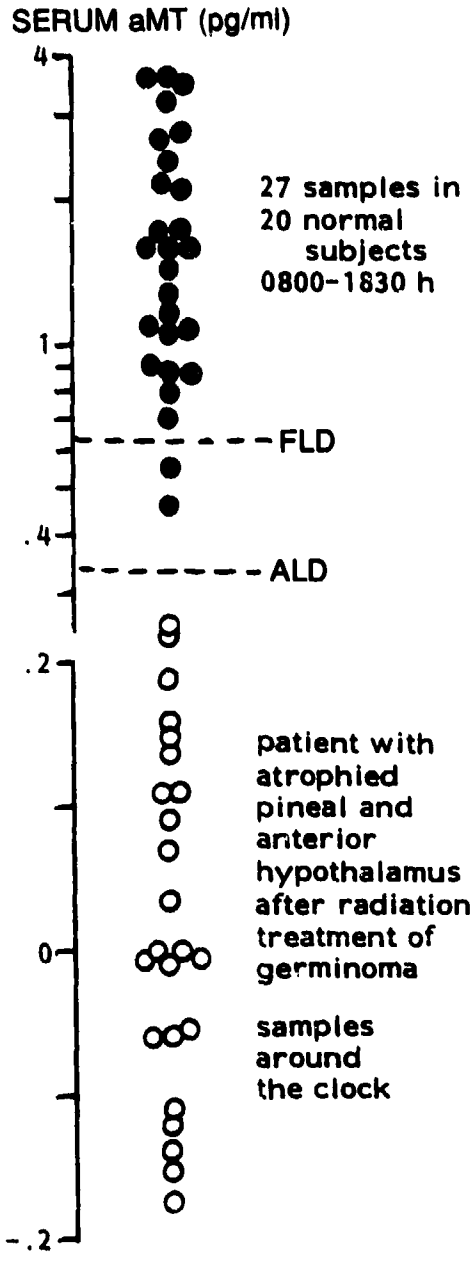

Fig. 10. Daytime samples from healthy subjects 22–60 years old (closed circles) compared with all those samples of the pineal atrophy patient (open circles) from Figure 9 (curve fit projected to negative aMT values for cpm above the fitted zero-standard cpm). ALD, analytic least detectable. FLD, functional least detectable taken as 0.62 pg/ml.

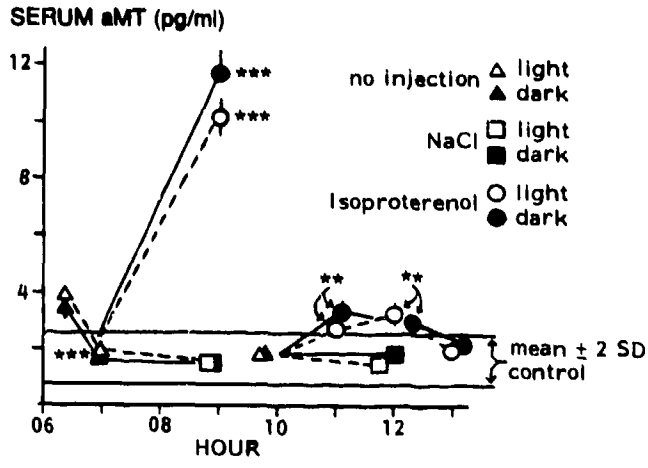

Fig. 11. Effect of a single 1 μg/g subcutaneous injection of isoproterenol (ISO) on Syrian hamster serum aMT in the early light phase. Injection was at either 0700 or at 1000 (not both). Open symbols and dashed lines indicate sampling after the usual lights-on (0600) had occurred. Closed symbols and continuous lines indicate sampling in novel morning darkness (lights-on at 0600 had not occurred). The control range shown is based on all samples from hamsters (not injected with ISO) taken after 0800. Within either light or novel dark: $***P < 0.001$ (0620 vs. 0700, 0900 ISO vs. NaCl); $**P < 0.01$ (1100 and 1200 ISO vs. 1200 NaCl). There were no significant differences between light and novel dark.

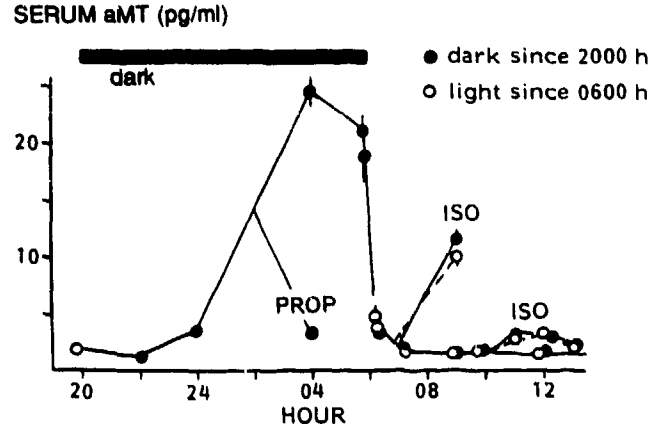

Fig. 12. Nocturnal serum aMT plotted with the data from the last figure. PROP, propranolol 1 μg/g given subcutaneously at 0200.

tion, and with only three BACV-aMT coordinates, only a tentative interpretation is warranted. A comparison [Strassman et al., 1989] of plasma aMT estimates in 43 samples between this RIA and the GC-MS assay of Lewy and Markey revealed a correlation that was almost entirely due to the approximately 30% of the values above 7 pg/ml on GC-MS (all of these being above about 18 pg/ml on RIA). The other samples ranged 1–7 pg/ml on GC-MS but 10–39 pg/ml on the RIA. About five of these values may have been below the RIA ALD and assigned the ALD value for the RIA (10 pg/ml). RIA values between 24 and 30 pg/ml (13 values on the plot) were associated with GC-MS values 2–27 (2–12 in all but two) pg/ml. If the results from the procedure with the lower values (GC-MS) can be taken as the comparison standard, then the data are compatible with an RIA FLD of about 23 pg/ml, below which RIA variation from the regression line at a given GC-MS value appeared increased. How-

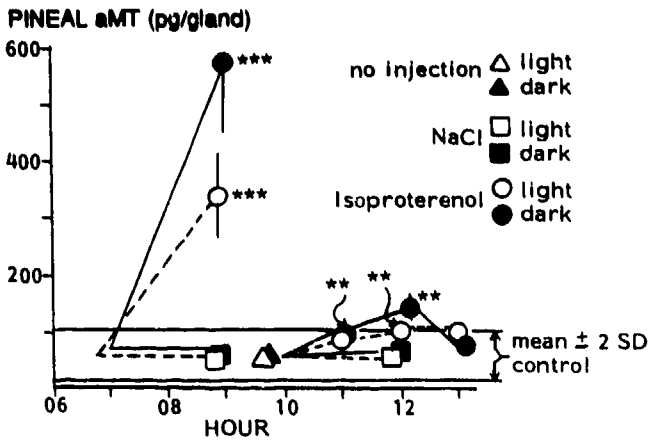

Fig. 13. Effect of isoproterenol (ISO) on pineal aMT content for the animals of Figure 11 in which pineal aMT was determined. $***P < 0.001$, $**P < 0.01$, ISO vs. respective NaCl. No differences were detected between light and novel dark. Control range based on animals not receiving ISO and sampled after 0800.

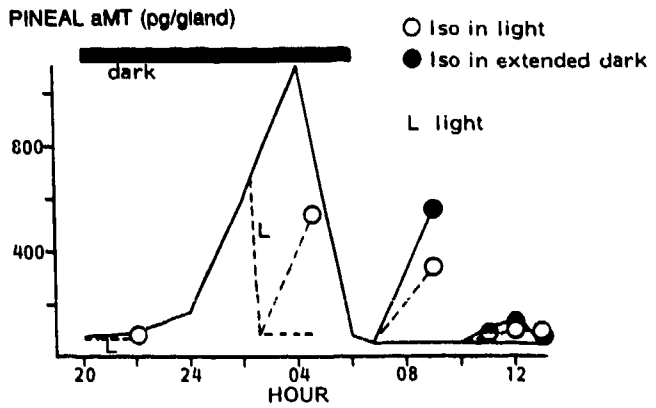

Fig. 14. Nocturnal Syrian hamster pineal aMT content from previously published data [Vaughan and Reiter, 1987; Vaughan et al., 1985] plotted along with the morning data of the present experiment from the Figure 13. ISO, isoproterenol (1 μg/g) injected subcutaneously in the morning or during short light exposure (L) at night. The nocturnal pineal data are from hamsters other than those providing the nocturnal serum data of the present experiment (Fig. 12).

ever, the functional error profile for GC-MS at a spectrum of aMT levels is also not known and may have contributed to the scatter in the data, presumably more so below some aMT level. That study interpreted results of daytime levels (for a small effect of exercise) but without assessing whether the results were below a FLD in either assay.

A modification of the above RIA has been used [Webley et al., 1985; Berga et al., 1988; Cagnacci et al., 1992] in which the sample volume (250–300 μl), amount of antibody, and reaction volume are reduced, and the reaction occurs in the scintillation vials. An ED_{50} of about 55 pg/ml and ALD's of 2, 10, and 6.5 pg/ml have been reported. Human day serum was about 16 pg/ml in one report or usually less than the ALD in another. The Kd for the antibody under these conditions was given as 17.6 pM. We have employed this (Stockgrand) antibody in our laboratory [Vaughan and Pruitt, 1993] by extracting 250-μl samples and standards (the latter in buffer) using a procedure including all the steps in Table 1, except that the initial antibody dilution was 1:3000 (50 μl, ¼ that usually used for this antibody in the original procedure), ^{125}I-aMT (instead of ^{3}H-aMT) was the tracer, the buffer was the originally-described tricine but at pH 7.0 for optimal binding, and the carrier-second-antibody system was in higher concentration (optimized). The ED_{50} was 28 pg/ml and the ALD 2.8 pg/ml (at zero-standard B/B_0 minus 2 SD, giving a B/B_0 of 95%). Further reduction in amount of antibody (presumably to increase sensitivity) was not possible, because the B_0) was already 28%. There were 17 daytime human samples (0.4–3.7 pg/ml in the currently reported G280 assay) available for comparison with this modified Stockgrand antibody procedure. There was 100% assay recovery in both procedures. All values were higher with the latter procedure, in which nine registered above 4 pg/ml, up to 9.6 pg/ml (data not shown). We did not determine the FLD. We found a Kd of 6.0 pM for ^{125}I-aMT.

Another procedure [Claustrat et al., 1984; Brun et al., 1985] utilizes diethylether extraction of 300-μl samples and a radioiodinated aMT analogue as tracer. The ED_{50} appears to be 100 pg/ml, and the ALD is reported to be 5 pg/ml. Human day serum values appeared to be 5–10 pg/ml. The procedure of Tiefenauer and Andres [1984] was originally described in buffer aMT standards without extraction and also utilizes an iodinated aMT analogue as tracer. The ED_{50} was approximately 400 pg/ml aMT and the Kd (from the reported Ka) was 1.6 (10^{-9}) M for the analogue and 8.3 (10^{-10}) M for ^{3}H-aMT. The method sold as kits (Tecova AG, Wohlen, Switzerland; Euro-Diagnostics, Apeldoorn, Holland) calls for extraction of 500 μl samples in diethylether or chloroform. Reports citing the original description and/or a kit [Demisch et al., 1988; Bieck et al., 1988; Khoory and Stemme, 1988; Tortosa et al., 1989; Souêtre et al., 1989; deLeiva et al., 1990; Souêtre et al., 1990] have involved extraction and ALD's of 5 or 10 pg/ml. Human day serum has ranged from less than 5 pg/ml to means of 7–14 pg/ml or 25 pg/ml.

The assay of Vakkuri and colleagues [1984], which has been used by others [Kauppila et al., 1987; Kivelä, 1991], utilizes 2-^{125}I-aMT as tracer

(as does the presently reported G280 assay), but extracts 1 ml serum in chloroform for an ED_{50} of about 45 pg/ml. The ALD was reported to be 4 pg/ml and human day serum ranged 4–15 pg/ml and had a mean value at 1100 of 9.7 pg/ml in one report [Kivelä, 1991]. In the latter report, three BACV values (10–24%) were given over a range of aMT compatible with a FLD of about 10 pg/ml.

An assay kit marketed by Nichols Insititute, San Juan Capistrano, California, involves diethylether extraction of 500 µl serum or plasma for an ED_{50} of 25 pg/ml and an ALD of 3 pg/ml. The tracer (aMT or analogue not specified) is radioiodinated. Of 21 human day serum samples, one registered 1.7 and the others 5–15 pg/ml (telephonic communication with Nichols in-house representative). An ELISA kit (ALPCO, Windham, NH) is available in which an aMT analogue containing a tracer enzyme competes with 50 µl unextracted sample for an antibody attracted to an immobilized second antibody. The ALD is stated to be 2.6 pg/ml and the ED_{50} about 148 pg/ml, and human day serum apparently ranged from undetectable to 15 pg/ml. Independent evaluation of these kits is needed for assessment of their performance.

Another kit (Elias USA, Osceola, WI) utilizes a radioiodinated aMT analogue in an RIA directly with 200 µl unextracted EDTA plasma for an ED_{50} of 28 pg/ml and an ALD of 1.5 pg/ml. A comparison (assay brochure) of human plasmas with the Lewy and Markey GC-MS method shows a correlation, but this appears entirely due to samples above 22 pg/ml RIA (above 15 pg/ml GC-MS). Of the 45 other plotted points, those from daytime were not identified. At RIA 2.5–4 pg/ml (well above the ALD), the ten plotted values for GC-MS ranged 0–14 pg/ml, and samples with RIA at 15–18 pg/ml (5 points) ranged 2.5–17 on GC-MS. At 12.5–15 pg/ml GC-MS, the 7 points ranged 3–21 on RIA, and samples with GC-MS at 2–4 pg/ml (18 points) ranged 2.5–18 pg/ml on RIA. It is not possible to determine whether the highly variable results below about 20 pg/ml stemmed from one or both (in combination) comparison procedures, though this extreme variation occurred at 10–15-fold above the ALD of both procedures. Method-related sources for this variation would only be identified if samples with the same aMT content at the levels of interest were assayed in multiple assays with both procedures to see if repeated results on the same sample show the same large variation in either procedure. This would allow a precision-based FLD to determine below what level either procedure gives a result not interpretable as representative of the procedure.

The gas chromatography negative chemical ionization mass spectrometry (GC-MS) procedure of Lewy and Markey [1978] [Lewy, 1983; Lewy et al., 1985; Lewy, 1985; Lewy et al., 1987; Lewy et al., 1992; Sack et al., 1992] has the lowest ALD reported so far, though the sample volume is 1 ml. The within-assay analytic parameters used to define threshold criteria for the ALD were not explicit, though it is mentioned [Lewy and Markey, 1978] that a judgement of the presence of the more intense ion peak above background noise may be aided by specificity parameters such as the presence of the less intense ion peak, peak symmetry, and absence of shoulders. Thus, the original concentration required to satisfy threshold criteria might be ascertained for a given recovery. For samples with less recovery, a greater pre-extraction concentration (i.e., a greater ALD) would be required for detection. Because of the extensive washing, extraction, and derivatization procedures, a deuterated aMT internal standard is added to the plasma sample (15–40 pg/ml) in sufficient quantity to provide another ion peak to determine recovery. This procedural recovery was said to vary as much as 5-fold [Lewy and Markey, 1978], which would mean that the pre-extraction ALD (reported as 1 pg/ml at an unspecified recovery) might vary greatly among plasma samples. From the description given, it also seems likely that a small signal above a valid ALD (the signal itself being accepted as qualitatively representative of some aMT from its specificity earmarks) might give a quantitative value containing a component of noise or error from random, procedural, or nonspecific serum factors, any of which may vary among samples. Then, at higher concentrations, the physicochemical parameters begin to provide specificity presumably for almost all of what is quantitated as aMT, providing the powerful asset of this important procedure. Though a functional assement of these intervening levels (e.g., with a FLD threshold) is not given, nadir daytime human plasmas have registered variously 1.5–4.9, 2–10, and a mean of about 7 pg/ml taken from a plot.

Use of a FLD (precision-based detectability) would not be restricted to RIA methodology (as much as are the ED_{50} and the B/B_0-determined ALD) and might provide a common reference frame for the lower limit above which aMT values can be taken reliably to represent the output of a given method. Comparisons between methods would be meaningful only if this assessment is available, because otherwise, the perturbingly large scatter from the regression line projected into the lower daytime range cannot be partitioned according to

method in the comparison. In the higher ranges, for methods capable of detecting the nocturnal surge, their outputs are expected to be correlated anyway. Previously, the FLD has not been applied intentionally to aMT assays. However, when BACV values were given for RIA's in a way to afford a tentative FLD estimate, they have been a little above twice the conservative estimate of the ALD.

The present G280 assay utilizes a smaller sample volume than almost all other assays and gives a FLD (0.62–0.80 pg/ml) about twice the ALD (0.33 pg/ml), the former well below even the ALD of other RIA's. Normal human daytime samples in our assay so far have been above the ALD and most above the FLD, so far not extending above 4 pg/ml. In other RIA's, human day values have either been quite higher and/or not in the usable range of the assay. Our least detectable (either as ALD or FLD) and daytime nadir seem to register lower than reported for the GC-MS method. Some of this smaller difference in daytime nadir might stem partly from use of different subjects in different locations.

Daytime young adult Syrian hamster (SH) samples have averaged about 25, 17, or 30 pg/ml in our modified Rollag antibody assay [Vaughan and Reiter, 1986; Vaughan et al., 1986b]; 5–20 or 18–20 pg/ml in the Pang assay [Brown et al., 1981; Reiter et al., 1982; Pang and Tang, 1983; Gibbs and Vriend, 1983]; 10–100 in the procedure of Brown et al. [1985]; and 22 pg/ml with the Stockgrand antibody [Reiter et al., 1991]. These values are all several fold above those assays' ALDs. A modified Claustrat procedure [Pévet et al., 1989], perhaps with a different antibody, gave mean values ranging 20–60 pg/ml in SH during the day. On the other hand, our present results show mean daytime normal SH serum aMT of about 1.8 pg/ml, with a range for individuals similar to that in normal humans.

The fall of SH serum aMT near the end of the dark phase appears entrained, not gated, since it was not different if darkness was suddenly extended past the end of the night. Even after serum aMT (present results) and pineal aMT [Vaughan et al., 1985] fall to daytime levels, the ability of subcutaneously injected ISO to simulate pineal and serum aMT persists in SH for several hours. Unlike the nocturnal surge itself, this response to a beta-adrenergic stimulus is not blocked by the acute presence of light. By 4 hr into the light phase, this response is already markedly attenuated. Thus, the fall of the nocturnal surge and the fall of beta-adrenergic responsiveness are controlled endogenously but are several hr out of phase. The persistence of a beta-adrenergic aMT response in the early light phase suggests that the preceding fall-off of the nocturnal aMT surge may be produced by endogenous withdrawal of the adrenergic drive to the pineal that is present in the second half of darkness and that can also be withdrawn artificially by propranolol administration.

The aMT response to either the endogenous neurotransmitter (producing the normal nocturnal aMT surge) or injected ISO (inducing a secondary surge at night after acute inhibition by light) requires new protein (N-acetyltransferase, NAT) synthesis from stimulation of RNA production in the SH [Gonzalez-Brito et al., 1990]. However, a dose of actinomycin D (ACTD) given in close proximity before late dark phase-ISO injection after acute light exposure (but not ACTD given earlier) apparently failed to inhibit the pineal aMT response to ISO. Cycloheximide was inhibitory at either time. Thus, one must consider that ISO injected at night after acute light might exert its effect at a posttranscriptional but still pretranslational site, if recent (e.g., nocturnal) adrenergic tone has already stimulated transcription [Romero et al., 1975]. Such an action of ISO might explain the presently observed morning ISO response by its reengendering the effectiveness of mRNA left over from the nocturnal surge. Thus, later daytime unresponsiveness to relatively brief adrenergic stimuli to the pineal in SH might reflect nonpersistence of the stimulus past a long lag period required to replenish NAT mRNA after it is depleted from waning of the previous (more prolonged) nocturnal surge of pineal sympathetic tone [Gonzalez-Brito et al., 1990]. However, the available data do not yet exclude possible adrenergic responsiveness also of pineal transcription varying around the clock in this species, becoming minimal after a few hours of light, and rising before the nocturnal surge of NAT/aMT and shortly before the ineffective dose of ACTD in the previous studies [Gonzalez-Brito et al., 1990] could have become effective. Whether morning aMT responsiveness wanes from falling NAT mRNA stores and/or from falling responsiveness of new mRNA synthesis, this does not occur from an acute effect of morning light and is thus endogenously determined.

The close parallel between pineal and serum changes in aMT, even seen when aMT is low and the response to ISO is minimal, indicates that alterations of daytime circulating aMT can be measured in this assay with a demonstrated basis of reliability. This improvement (enabling use with daytime levels) may tentatively classify it as a second-generation assay. However, it is still possible that normal daytime levels may be shown to be even lower should there be developed a third-generation assay with greater sensitivity and specificity in the lower range manifested in part by a substantially reduced FLD.

Disclaimers

The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. Human subjects participated in these studies after giving their free and informed voluntary consent. Investigators adhered to AR 70-25 and USAMRDC Reg 70-25 on Use of Volunteers in Research. Research was conducted in compliance with Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and in adherence with the *Guide for the Care and Use of Laboratory Animals*, NIH publication 80-23, 1985 edition.

Acknowledgments

I thank SSG Reva Berry and Mr. Leonard Seraile for their expert laboratory work; Ms. Sandy H. Coggins for her excellent technical, statistical, and editorial contribution and preparation of this manuscript; and Drs. D. Kennaway, B. A. Pruitt, Jr., and, especially, A. D. Mason, Jr. for their scientific advice.

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